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# Heat Shock Protein Induction in *Montastraea faveolata* and *Aiptasia pallida* Exposed to Elevated Temperatures

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**Abstract.** Frequent widespread episodes of coral bleaching have made researchers aware of the sensitivity of reef corals to moderately elevated temperatures and led us to investigate mechanisms of temperature stress tolerance in this group. One such mechanism may be the induced synthesis of heat shock proteins (hsps), which have been shown to play a role in thermotolerance in other organisms. However, induced synthesis of hsps in scleractinian corals was not reported until recently.

Experiments were conducted in which *Montastraea faveolata* was exposed to high temperatures (up to 35°C) for short periods (2 h). Under the conditions tested, the corals produced seven different hsps with approximate molecular weights of 95, 90, 78, 74, 33, 28, and 27 kDa. Another zooxanthellate species, the sea anemone *Aiptasia pallida*, also synthesized hsps during temperature stress, but fewer and with different molecular weights (82, 72, 68, and 48 kDa) than those produced by *Montastraea*. It now remains to be determined whether hsps are involved in differences in thermotolerance and susceptibility to bleaching within and between the various species of *Montastraea*, and between species of reef cnidarians.

## Introduction

Heat shock proteins (hsps) are also referred to as "stress proteins" because their expression can be induced by a number of stressful conditions, only one of which is heat shock (reviewed in Craig, 1985). These proteins are highly conserved evolutionarily: they have been found in all phyla examined thus far, from bacteria and yeast to humans.

Several functions have been proposed for hsps, including the transport of newly transcribed proteins to the endoplasmic reticulum and mitochondria and involvement in the insertion of these proteins into these organelles (reviewed in Gething and Sambrook, 1992; Parsell and Lindquist, 1993). Hsps may also play a role in translation and, during stress, may interact with unfolding (denaturing) proteins to either keep them in a refoldable conformation or target them for degradation (Welch, 1992). The production of hsps has also been correlated with the acquisition of enhanced thermotolerance (Parsell and Lindquist, 1993). Because of their important functions in normal metabolic and repair processes, moderate amounts of many hsps are present in unstressed cells at all times, but synthesis increases after a stress. In some cases, this increase in hsp synthesis is accompanied by a decrease in the production of other non-hsp proteins (Parsell and Lindquist, 1993).

Interest in the effects of elevated temperature on scleractinian reef-building corals and their ability to produce heat shock proteins has increased with recent frequent, widespread episodes of coral bleaching (Glynn, 1991; Williams and Bunkley-Williams, 1990). Corals have a complex symbiotic relationship with photosynthesizing dinoflagellates, called zooxanthellae, that are contained within the gastrodermal cells of corals. Bleaching results from the loss of the zooxanthellae, the degradation of chlorophyll pigments within zooxanthellae, or a combination of both. Many conditions have been shown to induce bleaching or are implicated in its production (reviewed in Williams and Bunkley-Williams, 1990), but the

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condition most often correlated with recent bleaching events is prolonged exposure to moderately elevated temperatures.

Hsp induction has been reported in a number of cnidarians. Two thermotolerant species of the hydrozoan *Hydra* were found to increase the synthesis of hsp60 when exposed to elevated temperature or to the chemical inducers sodium azide and cadmium chloride; six more thermosensitive species failed to produce this hsp (Bosch *et al.*, 1988). In the scyphozoan jellyfish *Aurelia aurita*, six hsps were identified when various developmental stages were exposed to elevated temperatures for a few hours (Black and Bloom, 1984). An anemone, *Anemonia viridis*, produced six different hsps in response to elevated temperatures or copper chloride (Miller *et al.*, 1992). Hsp70 was found in the coral *Goniopora djiboutiensis* (Sharp *et al.*, 1994), and other reports of hsp production in zooxanthellate cnidarians have been presented at recent scientific meetings.

The objective of this research was to determine whether the Caribbean reef coral *Montastraea faveolata* (Ellis and Solander)—formerly *Montastraea annularis* (Knowlton *et al.*, 1992; Weil and Knowlton, 1994)—produces hsps; and if so, which ones. Individual colonies and sibling species of this coral have various degrees of susceptibility to temperature-induced bleaching (Gates, 1990; Szmant and Gassman, 1990; Black, 1993). Samples of this coral were subjected to acute exposure to higher temperature followed by variable recovery periods at control temperature and then tested for elevation of hsp synthesis. Sea anemones, *Aiptasia pallida* (Verrill), were also used to work out techniques and to illustrate hsp production by another zooxanthellate cnidarian species. The exposure conditions tested for each species are summarized in Table I.

## Methods and Materials

### *Collection and maintenance of the animals*

Multiple cores from large colonies of *Montastraea faveolata* were collected from a depth of 5–7 m on a reef near Joulters Cay (25° 19′ N, 78° 05′ W), north of Andros Island, Bahamas, in 1988 and 1989. A pneumatic drill fitted with a hole-saw and powered by compressed air from a scuba tank was used to obtain short cores, 5 cm in diameter, with live tissue only on the top. The corals were used in nondestructive and nonstressful laboratory experiments involving temperature and light exposure and then returned to uncontrolled ambient laboratory conditions for several months before the start of these experiments.

Sea anemones of the species *Aiptasia pallida* were obtained from holding tanks at the Florida Keys Marine Laboratory on Long Key in February 1993. They were allowed one week to acclimate to laboratory conditions before experiments were started.

Specimens were maintained in flowing, sand-filtered seawater at ambient seawater temperature (*ca.* 28°C for the corals used in experiment 1, 20°–24°C for the corals and anemones used in experiments 2–4, Table 1) under cool-white fluorescent or metal halide lights (*ca.* 200 $\mu$Ein $m^{-2}$ $s^{-1}$ of light) on a 14:10 hour light:dark schedule. Thawed frozen brine shrimp were provided as food once a week.

### *Incubation procedures*

For all experiments, the organisms were incubated in filtered (Whatman GF/A) seawater with 5 $\mu$Ci/ml of either $^{3}$H-leucine (156.0 or 179.6 Ci/mmol specific activity) or $^{35}$S-methionine/cysteine (1141.0 Ci/mmol specific activity) purchased from New England Nuclear.

Incubation was either in glass dishes with gentle aeration in a dark incubator or in covered beakers in a shallow water bath. The specimens were placed in the containers with the radiolabeled amino acid at the beginning of the temperature exposure, left there for the duration of the recovery periods of each experiment, then rinsed three times with filtered seawater (to remove excess radiolabel before processing). Each treatment group contained two or three corals or four anemones, but anemones were pooled for processing.

**Table I**

*Summary of the animals, conditions, and isotopes used in the heat shock experiments*

| Exp. no. | Species | Control temp. (°C) | Treatment conditions | Recovery conditions | N per treatment* | Isotope |
|---|---|---|---|---|---|---|
| 1 | *Montastraea faveolata* | 28 | 31°C: 1 week, 34°C: 2 h | none | 2 | $^{3}$H |
| 2 | *M. faveolata* | 22 | 27, 29, 31, 33, 35°C: 2 h | 22°C: 2 h | 3 | $^{35}$S |
| 3 | *M. faveolata*<br>*Aiptasia pallida* | 22 | 33°C: 2 h | 22°C: 4 h | 2 corals<br>4 anemones | $^{35}$S |
| 4 | *A. pallida* | 22 | 27, 29, 31, 33, 35°C: 2 h | 22°C: 2 h | 4 | $^{35}$S |
| 5 | *A. pallida* | 22 | 33°C: 2 h | 22°C: 0, 1, 2, 4, 24, 48 h | 4 | $^{35}$S |

* Because of their small size, tissues of sea anemones were pooled for analysis.

*Tissue preparation*

Coral tissues were removed from the skeletons with a jet of 0.1 *M* Tris (pH 6.8) sprayed through an airbrush; resulting tissue slurry volumes ranged from 3 to 13 ml. Preparations were kept on ice throughout processing. The tissue slurry was homogenized and centrifuged (850 × *g*, 15 min, 4°C) to separate the coral animal fraction (supernatant) from the zooxanthellae (pellet). Anemones were ground in 2 ml of cold Tris buffer (0.1 *M*, pH 6.8) in a glass tissue grinder with a Teflon-tipped pestle. Zooxanthellae and unbroken cells were removed by centrifugation (6600 × *g*, 20 min, 4°C).

In the first experiment, the supernatants were centrifuged again at 12,000 × *g* (15 min, 4°C) to remove cellular debris and larger organelles (Ford and Graham, 1991). To 100 μl of the latter supernatants was added 50 μl of 2X protein loading buffer [PLB; 0.125 *M* Tris, 20% glycerol, 2% 2-mercaptoethanol, and 4% SDS (Gallagher and Smith, 1991)]. The amount of radioactivity in a subsample of each supernatant was measured with a Beckman model LS180 scintillation counter. The remainder of each sample was boiled for 1 min and frozen at −80°C.

In the later experiments, proteins were precipitated from the supernatants with 8% trichloroacetic acid (TCA). Proteins were pelleted by centrifugation (12,000 × *g*, 20 min, 4°C). The pellets were rinsed three times with 8% TCA and three times with acetone to remove TCA-soluble and acetone-soluble contaminants, and then redissolved in 1 ml of PLB. A subsample was removed for scintillation counting and the rest was boiled and frozen at −80°C.

*Gel electrophoresis and autoradiography*

Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% running gel; 3% stacking gel). Equal amounts of radioactivity per sample (approx. 400,000 dpm) were loaded in each lane, and protein molecular weight standards were run in parallel. The electrophoresis buffer consisted of 0.025 *M* Tris, 0.192 *M* glycine, and 0.1% SDS (Laemmli, 1970). After electrophoresis, gels were fixed (50% methanol, 5% glacial acetic acid, 30 min), rinsed in water (three times, 10 min each), incubated in a solution of 10% salicylic acid and 3% glycerol (20 min), and vacuum dried at 80°C (2 h.). Autoradiograms were produced by exposing Kodak X-Omat AR film to the dried gels for several days at −80°C. The approximate molecular weights ($M_r$) of protein bands on the autoradiograms were estimated by comparison with the positions of molecular standards on the gels.

Because the gels were loaded with equal amounts of radiolabel rather than equal amounts of protein, the results provide us with a relative comparison of how radiolabel was incorporated within each treatment, but do not allow us to compare rates of synthesis of the various hsps between treatments.

*Densitometry*

The autoradiogram from experiment 4 (Fig. 2A), in which specimens of *Aiptasia pallida* were incubated in a temperature series from 22° to 35°C (Table I), was analyzed with densitometry to compare quantitatively the densities of the hsp68 and hsp72 bands from animals exposed to the various temperatures. The densitometry image analysis system consisted of a NEC single-chip color video camera to produce the image, a Targa + 64 frame-grabber board to capture the image, and the MOCHA software package (Jandel Scientific) to do the density scan of the image. Equal areas of each temperature lane were scanned, and the density values associated with each band summed. Figure 3 includes the density scans for each lane (A), and histograms that present the density and the percentage of total density of each hsp in each lane (B, C).

## Results

Exposure to elevated temperature increased the synthesis of several discrete proteins (hsps) in both corals and sea anemones. Each species produced its own characteristic set of hsps. The results of each experiment are summarized separately for each species.

*Montastraea faveolata (Fig. 1)*

*31° & 34°C vs. 28°C.* Corals exposed to 34°C expressed several hsps with $M_r$ of 33, 74, 78, 90, and 95 kDa. In this experiment, but not subsequent ones, there was reduced synthesis of other proteins such as a 40 kDa protein presumed to be actin and an unknown 99 kDa protein (Fig. 1a). The patterns of proteins synthesized at 28° and 31°C were similar and did not include any discernible hsp bands.

*27°–35°C vs. 22°C.* Hsp synthesis was tested at five temperatures from 27° to 35°C. No hsps were synthesized at 27°, 29°, or 31°C (not shown). The only hsps that were distinguishable in this experiment were hsp74 in the 33°C (faint) and 35°C (darker) treatments as well as two bands of *ca.* 27 and 28 kDa at 35°C (Fig. 1b). A repeat of the 33°C exposure experiment produced a pattern of hsps at 90, 78, and 74 kDa (Fig. 1c) similar to that seen before at 34°C.

*Aiptasia pallida (Figs. 2, 3)*

*27°–35°C vs. 22°C.* In this experiment, anemones were exposed to five different 2-h heat treatments (Fig. 2a). At 22° (control), 27°, and 29°C, the anemones did not appear stressed. At 31°C, however, the anemones were partially

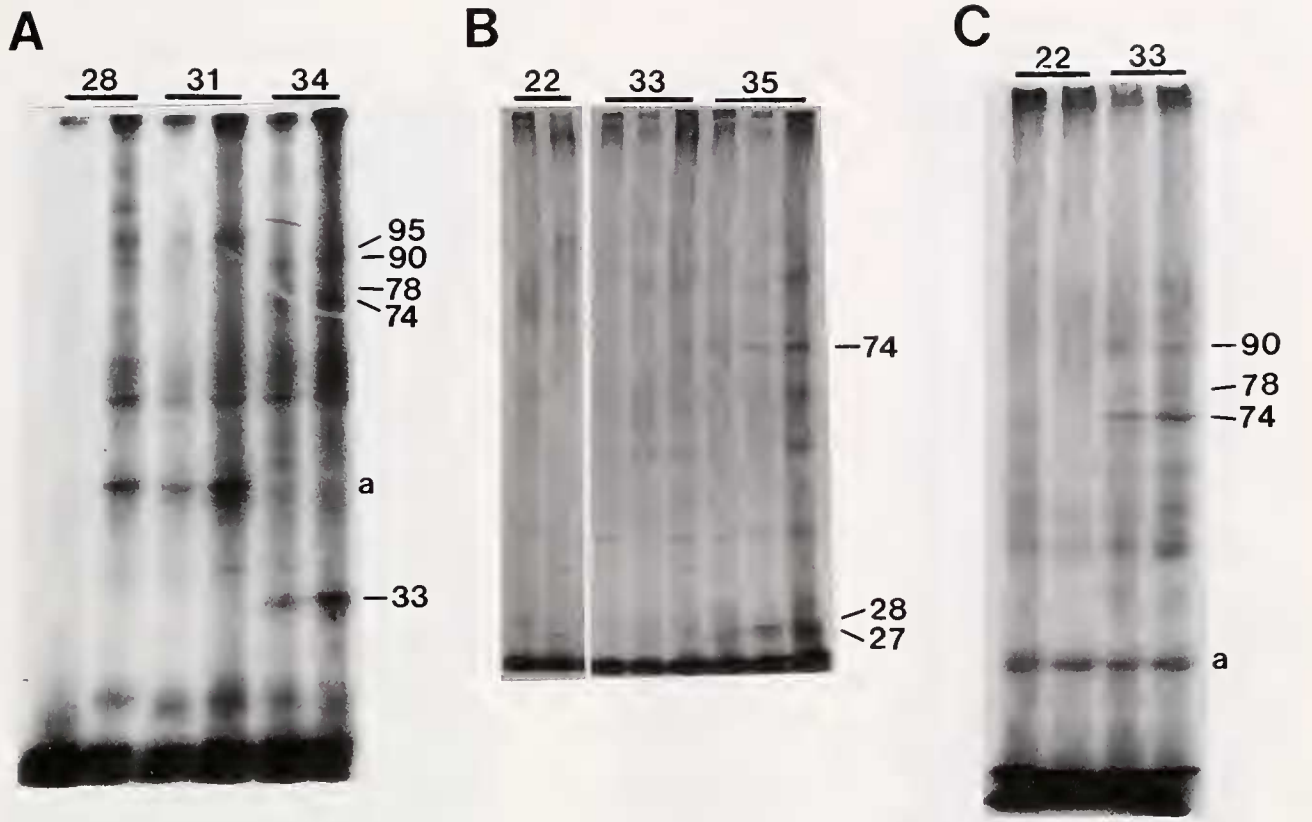


**Figure 1.** Autoradiograms showing proteins synthesized by *Montastraea faveolata* after exposure to various temperatures. (A) Synthesis after 1 week at 31°C or 2 h at 34°C compared to controls (28°C). The positions of hsps produced at approximately 95, 90, 78, 74, and 33 kDa are indicated. (B & C) Synthesis after 2 h at 33° or 35°C compared to controls (22°C). The positions of hsps produced at approximately 74, 28, and 27 kDa (B) and 90, 78, and 74 kDa (C) are indicated. Each lane represents the proteins of an individual coral, with two or three replicate corals per temperature treatment. a = protein presumed to be actin.

contracted during heat exposure, but recovered to normal appearance during the 2-h recovery period at 22°C. At 33° and 35°C, the anemones were severely stressed: all were totally contracted and some were lying on their sides. The 33°C anemones returned to normal appearance during the recovery period, but the 35°C anemones did not.

Only two hsps (68 and 72 kDa) could be distinguished visually in this experiment (Fig. 2A). The densitometry was used to quantify the amounts of radiolabel incorporated into each band with respect to the total amount of radiolabel in protein in each sample (Fig. 3). The density scans for each lane are presented in Figure 3A and the relative densities within the hsp68 and hsp72 bands in Figures 3B and 3C, respectively. The 72 kDa hsp was present as a faint band in the control (22°C) and 27°C anemones (2.8% of total density for each), with up to a 32% increase in the 29°, 31°, and 33°C animals (3.7%, 3.2%, and 3.3% of total, respectively), and then declined at 35°C (to 2.3% of total). Hsp68 was present at very low levels (1.9% of total) at the lowest two temperatures, increased by 30% to 50% at the intermediate temperatures, (29°, 31°, and 33°C: 2.6%, 2.4%, and 3.0% of total, respectively), and then increased dramatically by 280% at the highest temperature (35°C: 7.25% of total). In some experiments additional hsps were detected at 82 and 48 kDa (Fig. 2B), that were apparently induced to a lower extent than the hsps 68 and 72.

*33°C vs. 22°C, variable recovery period.* This experiment examined the turnover of hsps synthesized during the temperature stress and post-stress recovery periods. Increased amounts of hsps 68 and 72 (and 48 and 82, to a minor extent) were synthesized after a 2-h heat treatment at 33°C (comparison of lanes C and 0 in Fig. 2C; especially notice the increased density of the hsp68 and hsp82 bands in lane 0). Both of these hsps remained elevated for up to 24 h at 22°C (Fig. 2C), but were less evident in the 4-h and 48-h samples. Because of the experimental design (specimens were incubated with the radiolabel during both the stress and recovery periods), we cannot distinguish between radiolabeled hsps produced during the stress itself

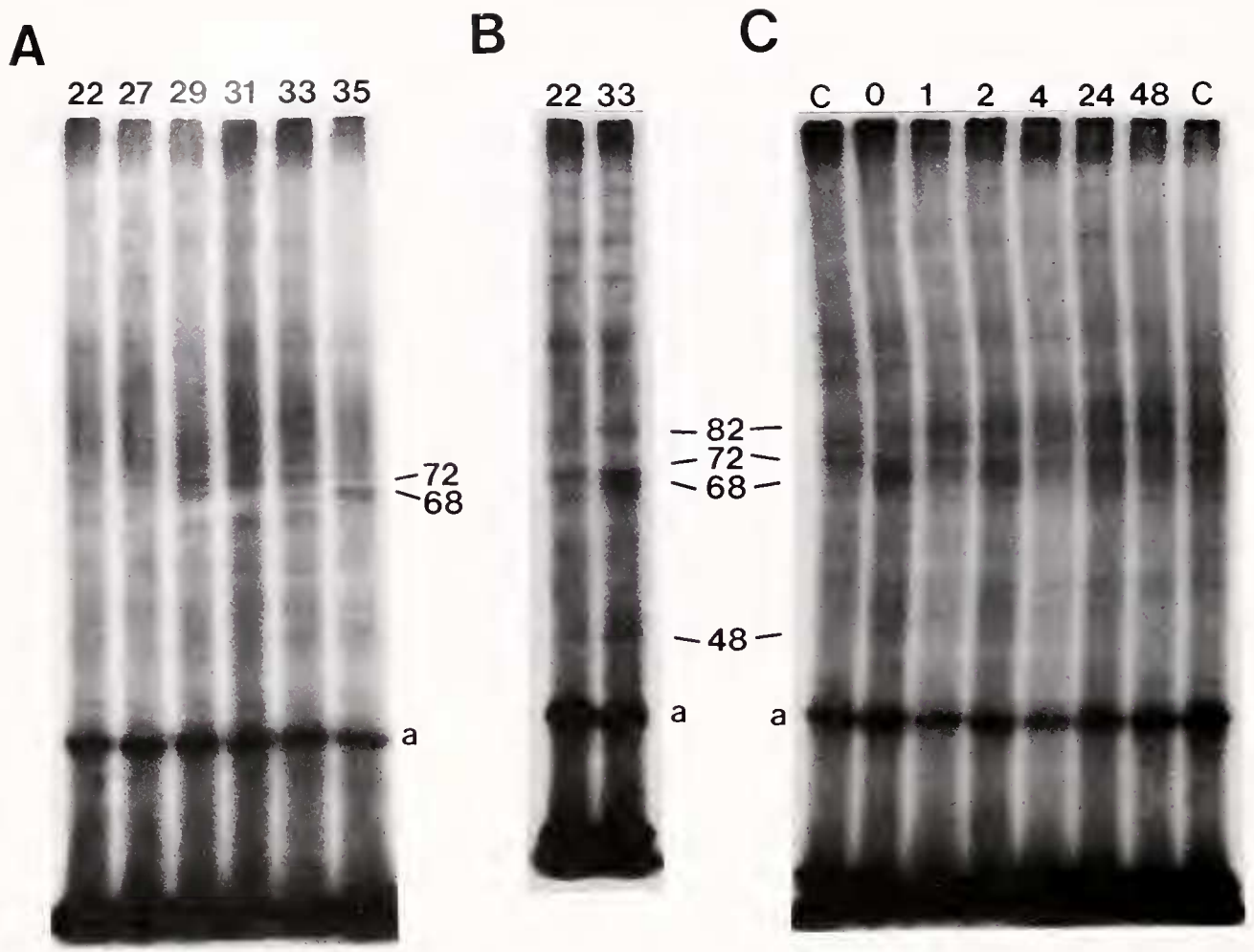


**Figure 2.** Autoradiogram showing proteins synthesized by *Aiptasia pallida* after exposure to various temperatures. (A) Synthesis after 2 h at 27°, 29°, 31°, or 35°C compared to controls (22°C). (B) Synthesis after 2 h at 33°C compared to controls (22°C). (C) Synthesis after 2 h at 33°C with variable recovery times (for 0, 1, 2, 4, 24, or 48 h) at 22°C compared to controls (22°C). Each lane contains a sample consisting of pooled tissues from four sea anemones. a = protein presumed to be actin. The positions of hsps produced at approximately 72 and 68 kDa (A) and 82, 72, 68, and 48 kDa (B, C) are indicated.

and subsequently during recovery. However, in other studies in which we have incubated reef corals with radiolabeled amino acids, the medium was depleted of more than 80% of the radiolabel within the first 3 h (FitzGerald and Szmant, 1988, in prep.). Thus, it is likely that most of the radiolabeled hsps (and other proteins) in the samples were synthesized during the stress period and the early hours of the recovery period.

## Discussion

Several hsps were found in both *Montastraea faveolata* and *Aiptasia pallida* after brief exposures to elevated temperature. Overall, hsp74 for *Montastraea*, and hsps 68 and 72 for *Aiptasia* were the dominant hsps produced in response to heat stress. In the latter species, hsp72 appears to be constitutive, with moderately increased synthesis after moderate temperature shock, and down-regulation at the highest temperature tested (35°C). In *Aiptasia*, hsp68 appears to be the major one produced with more extreme temperature stress. Given the near-background levels of this hsp in the control and 27°C samples, hsp68 may be inducible rather than constitutive.

Comparison of our results with those reported for other cnidarian species reveals a few common hsps and hsp families (Table II). According to Craig (1985), nearly all species produce hsps in the same three size classes: hsp90 (80 to 90 kDa), hsp70 (68 to 74 kDa), and small hsps (18 to 30 kDa). Variability regarding the number and size of hsps is generally greater for small hsps. Five of the cnidarian species studied produced one hsp in the 80 to 90

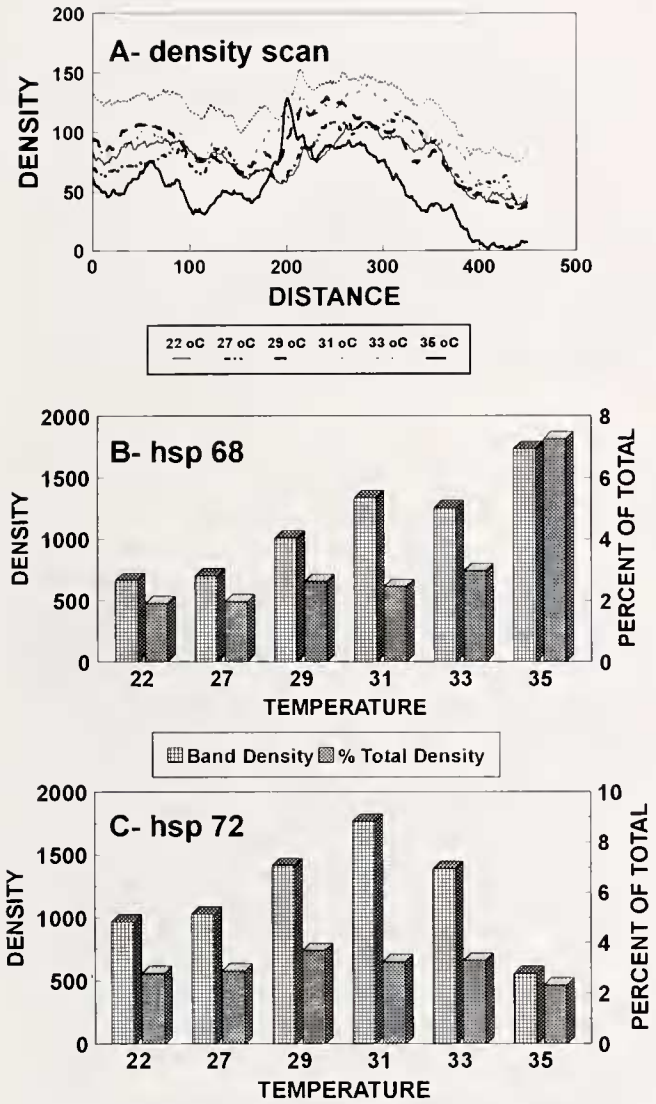


**Figure 3.** (A) Density profile along the central portion of each lane shown in Figure 2A, extending lengthwise about the mid-third of each lane. The scan width was about two-thirds the width of each lane. See text for further details. (B) The total density assigned to the hsp68 band of each sample, and as a percentage of total lane density. (C) Same as (B) but for the hsp72 band.

range, and all but *Hydra* produced one or more hsps in the 68 to 74 range.

As has been observed for other species, the number and relative quantities of hsps synthesized in our experiments increased with increasing severity of heat treatment. There was, however, variability between experiments in the amount and type of hsps produced, especially in *Montastraea*. Some of this variation may be due to individual differences in thermotolerance or ability to turn on hsp synthesis. This possibility should be addressed by repeating these experiments with greater replication. In addition, immunochemical techniques (*i.e.*, Western blots) should be applied to determine whether the hsps detected are members of the known hsp families. This approach has been used recently by Sharp *et al.* (1994) to identify members of the hsp70 family in a coral and a sea anemone (Table II).

Hsps in *Aiptasia* appear to have a moderate turnover rate. Radiolabeled hsps synthesized during and after the 2-h heat shock were still present 24 h after temperature exposure, but had apparently been degraded by 48 h after treatment. Experiments should be conducted to determine the duration of heat stress during which these animals can continue to synthesize hsps.

An attempt to determine whether zooxanthellae in *Montastraea* produce hsps failed because tissue homogenates of the zooxanthellae preparations contained too little radiolabel (4% or less) to allow detection of hsps. Although hsps have not yet been reported in zooxanthellae, the degradation and loss of pigments from the chloroplasts of zooxanthellae in bleached corals (Gladfelter, 1988; Kleppel *et al.*, 1989; Porter *et al.*, 1989; Glynn and D'Croz, 1990; Szmant and Gassman, 1990; Black, 1993) would indicate that the zooxanthellae are heat stressed and could also benefit from hsps.

In this study, we have shown that *Montastraea faveolata* can produce hsps in response to short exposures (several hours) to relatively highly elevated temperatures (≥33°C). Our experimental conditions differ from those associated with natural bleaching, which is thought to be caused by exposure to more moderately elevated temperatures (29°–30°C) for longer periods (several weeks to months) (Glynn and D'Croz, 1990; Black, 1993; Black and Szmant, in prep.). No hsp production was observed in corals exposed to moderately elevated temperatures (27°–31°C) for 2 hours to one week. In addition, the duration of our experiments was too short to expect any visible bleaching. Susceptibility of corals to bleaching varies between different species (Williams and Bunkley-Williams, 1990). The present results suggest that further work is warranted to determine whether hsps are involved in differences in thermotolerance and susceptibility to bleaching within and between reef cnidarian species.

## *Conclusions*

(1) Both *Montastraea faveolata* and *Aiptasia pallida* produce heat shock proteins in response to short periods of extreme but sublethal thermal stress. Most of the hsps produced appear to fall into a molecular weight range similar to those found in other cnidarian species. Western blots are needed to learn whether the hsps produced by *Montastraea* and *Aiptasia* belong to the same families as the hsps found in other groups. (2) The involvement of heat shock proteins in coral bleaching remains to be determined.

**Table II**

*Comparison of the heat shock proteins observed in six different cnidarian species*

| Species | Class | Molecular weight (kilodaltons) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Hydra attenuata*[a] | Hydrozoa | | 28 | | | | 60 | | | 80 | |
| *Aurelia aurelia*[b] | Scyphozoa | | | | 39 | 45 | 68 | 70 | | 83 | 93 |
| *Anemonia viridis*[c] | Anthozoa | 30 | 31 | 35 | 39 | | 69 | | | 82 | |
| *Anemonia viridis*[d] | Anthozoa | 28 | 29 | | | | | | | | |
| *Goniopora djiboutiensis*[d] | Anthozoa | | | | | | | 70 | | | |
| *Aiptasia pallida*[e] | Anthozoa | | | | | 48 | 68 | 72 | | 82 | |
| *Montastraea faveolata*[e] | Anthozoa | 27 | 28 | 33 | | | | 74 | 78 | 90 | 95 |

[a] Bosch *et al.* (1988).
[b] Black and Bloom (1984).
[c] Miller *et al.* (1992).
[d] Sharp *et al.* (1994).
[e] This study.

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